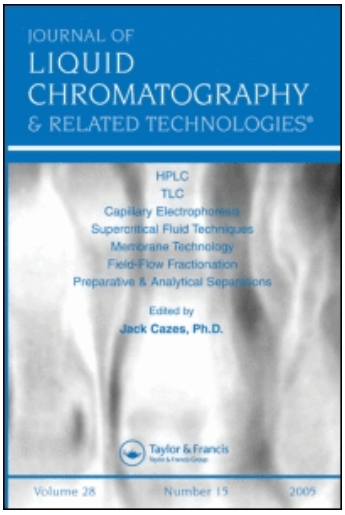

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Determination of Physostigmine in Plasma by Liquid Chromatography with Dual Electrode Amperometric Detection

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DETERMINATION OF PHYSOSTIGMINE IN PLASMA BY LIQUID CHROMATOGRAPHY WITH DUAL ELECTRODE AMPEROMETRIC DETECTION

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ABSTRACT

Physostigmine in plasma is determined after solid phase extraction. Ion-pair liquid chromatography is used for the separation and dual electrodes in a series mode are used for the detection. Physostigmine is first oxidized at +1.0 V and the reaction product formed is determined by reduction at +0.10 V versus Ag/AgCl. The solid phase extraction gives a recovery of 62 ± 6% (n=5) from plasma spiked with physostigmine to a concentration of 14 ng/mL. A detection limit of 0.5 ng/mL is obtained from a sample of 2 mL plasma.

INTRODUCTION

Physostigmine (Phy) (eserine) is an alkaloid extracted from the Calabar bean (Physostigma Venenosum) and it has long been used in ophtalmology (1). Today Phy is still mainly used as a

miotic (2), but also as an antidote for poisoning by anti-cholinergic drugs (3) and for reversing anti-cholinergic effects e.g. in connection with anaesthesia (4). Recently, its inhibiting effect on acetylcholinesterase has been investigated for the treatment of senile dementia in Alzheimer's disease (5) and for its possible prophylactic function in the defense against organofluorophosphate intoxication (6).

Analytical methods such as colorimetry (7), GLC (8) and TLC (9) have been applied for the determination of Phy in pharmaceutical preparations. For the low concentrations of Phy present in biological samples like blood, plasma and brain tissue, previous methods include an enzymatical (10) and several liquid chromatographic with UV (11-13) or electrochemical detection (14, 15). The most successful result in terms of detection limit has been reported by Whelpton and Moore (15) who used a silica column at pH 8.9. After alkaline extraction of 4 mL plasma, concentrating it to 60 µL and injecting 50 µL, it was possible to determine Phy in plasma at the 25 pg/mL level with a R.S.D. of 19.6% (n=5).

In this paper we report on a different approach to the development of a method for the determination of Phy in blood plasma. Phy is known to rapidly decompose in alkaline solution with the formation of the hydrolyzed products eseroline (Ese) and rubreserine (Rub) (16). Therefore we have avoided alkaline conditions. For the clean-up step we utilized an acidic solid phase extraction including ion-pairing. This is a modified version of the method described by Hsieh, Yang and Davis (12) and it offers a 20-fold pre-concentration of the sample. For the LC separation a reversed phase ion-pair system was chosen. Phy is positively charged in the pH region used with silica based bonded phase columns and ion-pairing provides symmetrical peak shape and high efficiency, further improved by the use of a slightly elevated temperature. Initial cyclic voltammetry studies revealed certain electrochemical properties of Phy which could be advantageously applied to dual electrode detection. The system was optimized by recording a hydrodynamic voltammogram (HDV), the use of three

working electrodes (see experimental section) and consideration of the influence of flow rate and spacer thickness on the amperometric response versus noise. The complete assay is evaluated in terms of linearity, recovery and detection limit.

EXPERIMENTAL

Instrumentation:

The LCEC system was a Bioanalytical Systems LC-304 with an LC-23A column heater and an LC-22A temperature controller (Bioanalytical Systems, West Lafayette, IN). Two LC-4B amperometric controllers were used for the dual electrode detection. The thin-layer cell was equipped with an experimental array of three series glassy carbon electrodes of which the two first were controlled at a common potential E_1 and the third electrode at a potential E_2. A Ag/AgCl reference electrode was used. The injector valve was a Rheodyne Model 7125 with a 20 µL injector loop and chromatograms were recorded on a dual-pen strip chart recorder (Houston Instruments). A Biophase Octylcolumn, 250 x 4.6 mm of particle size 5 µm, was used.

For cyclic voltammetry, a BAS 100 Electrochemical Analyzer was employed with an Auto Cell stand, a glassy carbon working electrode, a Pt wire auxiliary electrode and a Ag/AgCl reference electrode (Bioanalytical Systems).

Methods:

The C_{18}-SepPak cartridge (Waters) was pre-wet with 3 mL of methanol (redistilled) and 5 mL of water. One mL of 0.5% (w/v) sodium dodecylsulphate (SDS) (Sigma, St Louis, MO) was added to 2 mL of plasma and mixed before passing it through the SepPak. The

extraction column was then consecutively flushed with two 5 mL portions of water, 3 mL 60% methanol and finally 1 mL methanol, at a flow rate not exceeding 2 mL/min. The methanol fraction was evaporated under a gentle stream of N_2 at about 30°C and the residue was dissolved in 100 µL 40% acetonitrile. The sample was filtered on a microfilter system (Bioanalytical Systems) before injecting it onto the liquid chromatograph. The volume of sample was enough for duplicate injections when so desired.

The mobile phase consisted of 60% buffer pH 3.0, made up from 0.1 M sodium dihydrogen phosphate (Mallinckrodt, St Louis, MO) and 0.1 M phosphoric acid (85% Fisher, Fair Lawn, NJ), 40% acetonitrile (Omnisolv) and 1.0% (w/v) (34 mM) SDS. The flow rate was 2 mL/min and the column temperature was set at 35°C.

A stock solution of Phy (free base, Sigma) was prepared in acetonitrile with a concentration of 10 mM. This solution was stable for several months when kept refrigerated. All water used was deionized and filtered through a water purification system (Barnstead).

RESULTS AND DISCUSSION

Electrochemistry:

Cyclic voltammetry (CV) was run on Phy at pH 5, 6 and 7 to investigate the suitability of using electrochemical detection for an LC determination of this compound. The CV scans recorded at pH 5 at two different sweep rates are shown in Figure 1. In sweep A there is an oxidation peak at +0.80 V and on the reversal scan a reduction peak at +0.20 V. In sweep B, recorded with 5 times faster sweep rate, a reduction peak B appears at +0.70 V. On a second scan (not shown in figure) a new oxidation peak at +0.30 V is obtained in addition to the other three peaks. These results indicate an ECE mechanism where the oxidized product formed at the electrode

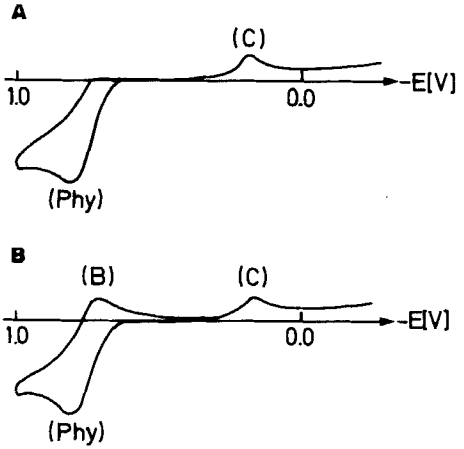

FIGURE 1. Cyclic voltammetry scans of 2.5 mM Phy in acetate buffer pH 5. Initial potential: 0 mV. Sweep direction: positive. Reversal potential: +1.0 V. Sweep rates: A, 50 mV/s; B, 250 mV/s.

surface undergoes a chemical reaction to yield another electroactive compound. In a pH 6 solution the same main characteristics appear in the CV scans, but with the potentials perturbed about 60 mV towards more negative values, indicating the involvement of one proton per electron in these electron transfer reactions. At pH 7 no reduction peak B can be found, not even at a fast scan rate of 1.0 V/s. A new reduction peak appears at a more negative potential, -0.1 V, indicating the formation of one more electroactive substance by some chemical reaction. Further investigations of these features were performed by coulometry and chronoamperometry and these results have been presented (17). The proposed electrochemical pathways are outlined in Figure 2. It was found that the number of electrons transferred are 2 in each process and that the irreversible chemical reactions occurring are favoured by an alkaline pH.

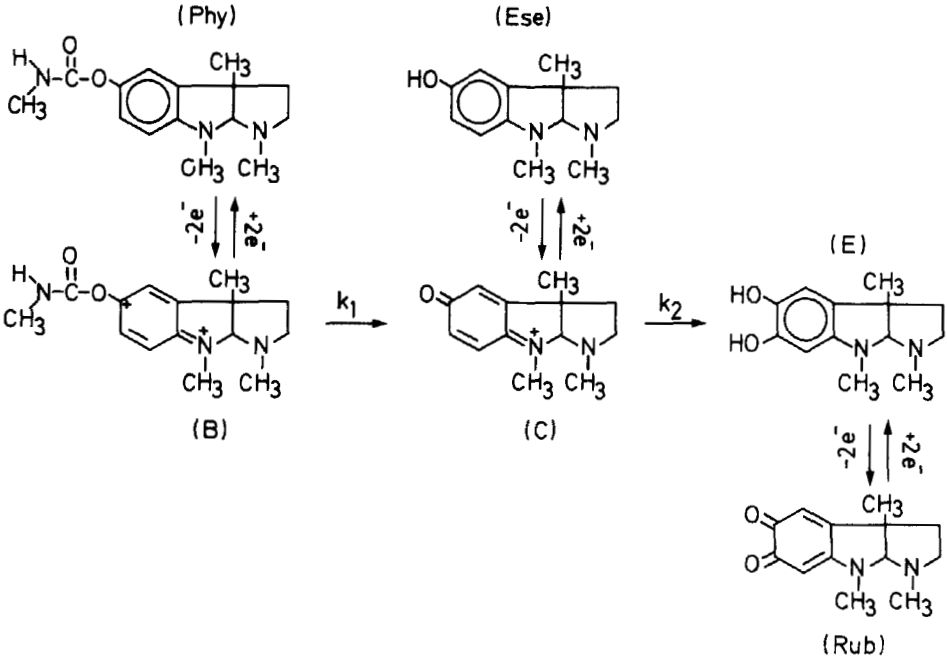

FIGURE 2. Electrochemical pathways of Phy.

Dual electrode detection:

Dual working electrodes in series were chosen for Phy detection. Phy is here oxidized upstream at a potential of +1.0 V. On the time scale of this experiment the main part of the oxidation product has converted into compound C (as shown in the scheme above) when the analyte reaches the downstream electrode where C can be reduced at the moderate potential of +0.1 V. What we gain by determining Phy at +0.1 V instead of at +1.0 V is low background current, yielding a stable baseline with improved signal to noise ratio, and higher selectivity, since very few compounds are reduced at +0.1 V compared to those oxidized at +1.0 V. An HDV was recorded at pH 3 by injection of Phy at each potential setting of E_2 in the range

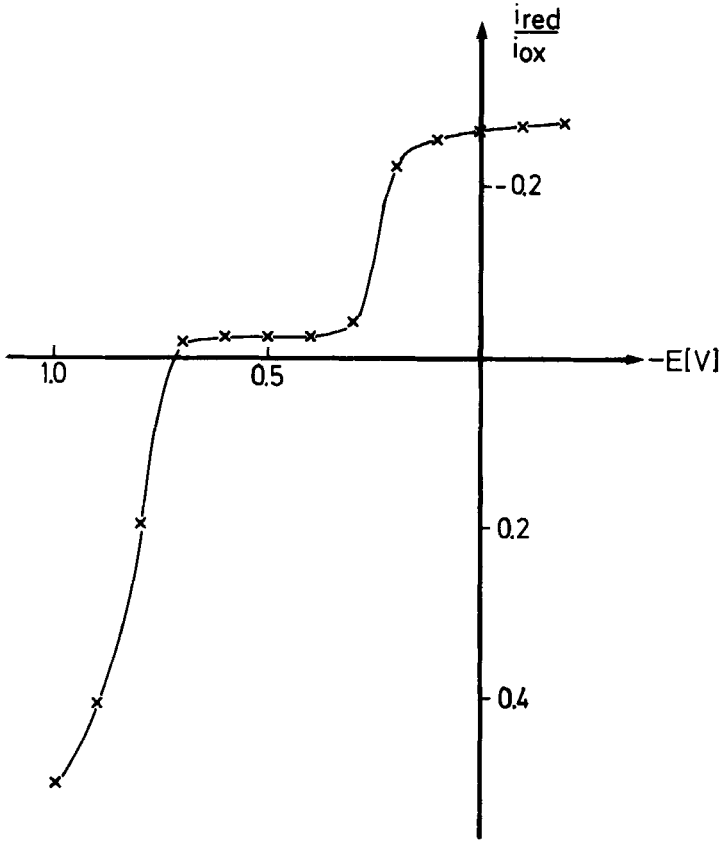

FIGURE 3. Hydrodynamic voltammogram of Phy recorded at pH 3. E_1= +1.0 V and E_2 is varied. The current ratio i_2/i_1 is calculated and plotted versus potential E_2.

-0.2 - +1.0 V while E_1 was held constant at +1.0 V (Figure 3). From this plot the optimal potentials were chosen to be E_1= +1.0 V and E_2= +0.1 V where the current is mass transport limited as indicated by the plateau on the HDV in these regions.

A small enhancement (15%) of the reduction peak current was obtained when a two-electrode cell was replaced by an experimental three-electrode cell with the two first electrodes connected and

held at +1.0 V to give a larger production of oxidized Phy. Another enhancement of the signal of about 20% was obtained when the flow rate was increased from 1 mL/min to 2 mL/min. Amperometric detection is flow rate dependent because of mass transport to the electrode surface (18). This is described by the equation for the mass transfer coefficient $h=1.467(D/b)^{2/3}(\overline{U}/A)^{1/3}$, where D is the diffusion coefficient, b is the thickness of the thin-layer channel, $\overline{U}$ is the average flow rate and A is the electrode area. The increase in flow rate did not have any adverse effect on chromatographic resolution and was considered preferable, resulting in only half the analysis time. The 25 cm octyl column with this flow rate and a column temperature of 35°C gave a backpressure of 2800 psi which is acceptable. A third improvement of the response was obtained when the spacer of the thin-layer cell was replaced with one of thinner dimension. Changing the spacer thickness from 125 µm to 50 µm improved the reduction peak height 2.4 times. This is in approximate agreement with the dependence of current on thin-layer thickness as described by the equation for the mass transfer coefficient given above. Noise levels were recorded at 1 nA full scale under both conditions and no difference was apparent. The response was found to be linear throughout the concentration range investigated, 10^{-8}-10^{-5} M Phy, with a correlation coefficient of 0.999996.

Chromatographic system:

Phy is an amine with a pKa_2 value of 8.0 at 25°C as determined by Christenson (19) and is therefore protonated and positively charged throughout the pH-range used with bonded phase silica columns. Ion-pair chromatography with some alkylsulfate or alkylsulfonate as the ion-pairing agent is often used satisfactorily in similar cases. This was initially tried with an ODS-column, but without satisfactory results. Generally, a low plate number was calculated, 50 - 1000, with low peak symmetry and

poor reproducibility. When the ODS-column was replaced by an Octyl-column, performance was improved considerably. Figure 4 illustrates this difference in performance using the same mobile phase with both types of columns. Despite the high concentration (34 mM) of SDS in the mobile phase, the dominating retention mechanism is by ion interaction rather than micellar chromatography (20). This is noted from the way in which retention increases with increasing concentration of SDS. The critical micelle concentration for SDS in water is only 8 mM, but the content of organic solvent in this mobile phase seems to prevent the formation of micelles. SDS was chosen preferably to any smaller alkylsulfate because Phy elutes later than all other major peaks in the plasma chromatogram avoiding interferences. This chromatographic system also offers a complete separation of Phy from its decomposition products Ese and Rub, as verified by injection of these compounds (21).

Plasma clean-up:

Since Phy is rapidly and irreversibly hydrolyzed in alkaline solution, the alkaline extraction used by Whelpton and Moore (15) was considered not desirable for the clean-up step. Instead the acidic solid phase extraction described by Hsieh, Yang and Davis (12) was tested. Their procedure was modified by us to the method described in the experimental section. This procedure gave a recovery of 62 ± 6% (n=5) from plasma spiked with Phy to a concentration of 14 ng/mL. Chromatograms of a blank, a spiked plasma and a standard are shown in Figure 5 a-c. Since the sample is concentrated 20 times during pretreatment the ionic strength of the sample is quite high and this gave rise to some problems with system peaks (22). However, it was found that a high concentration of ion-pairing agent to a large extent suppressed these peaks and this was taken into consideration when the mobile phase composition was optimized. When the recovery of Phy from spiked

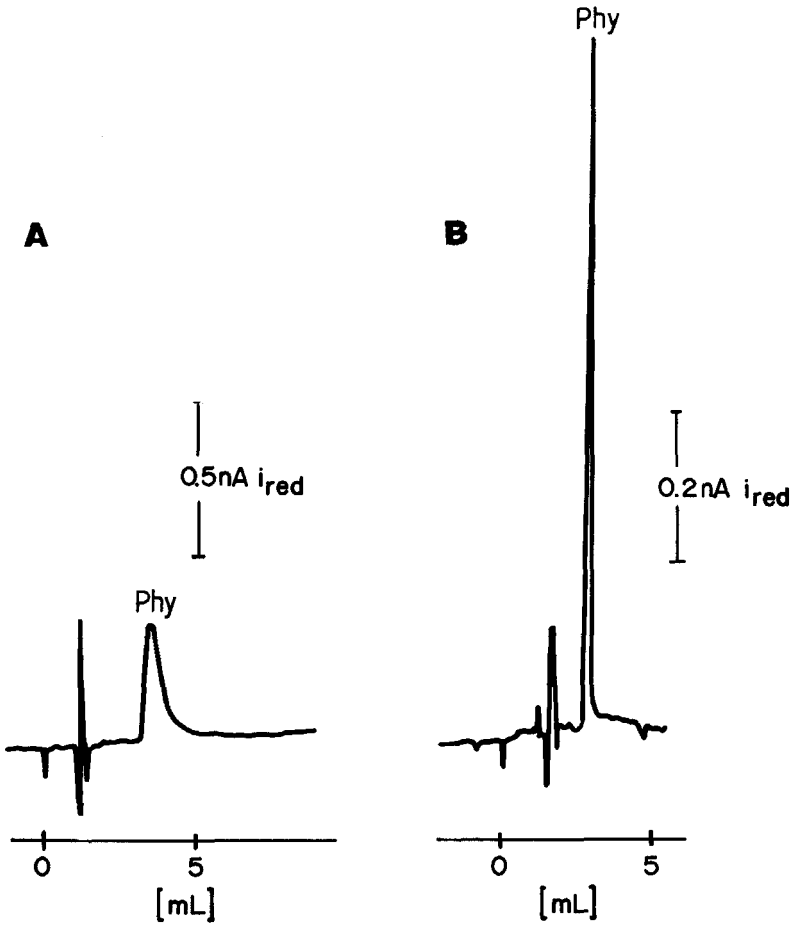

FIGURE 4. Chromatograms of 0.5 ng injected Phy on two columns of same dimensions and particle size. A, ODS column; B, Octyl column. Mobile phase: 40% acetonitrile, 60% 0.1 M citrate buffer pH 3.7, 5 mM octylsulfate.

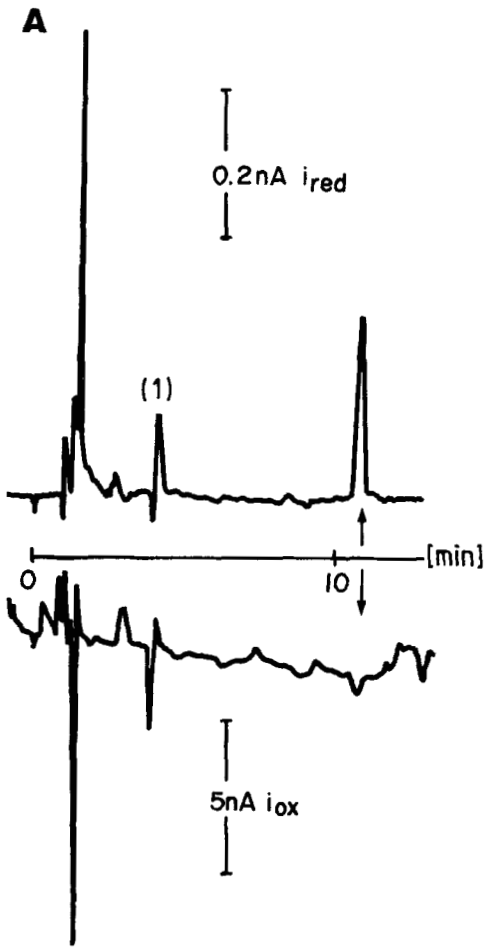

FIGURE 5. Chromatograms of: A, Standard, 0.5 ng injected; B, Plasma spiked with 14 ng/mL; C, Plasma blank. The retention time of Phy is indicated with an arrow. Peak (1) is a system peak and peak (2) appeared in some samples, but was adequately resolved from Phy. Chromatographic conditions as in experimental section.

(continued)

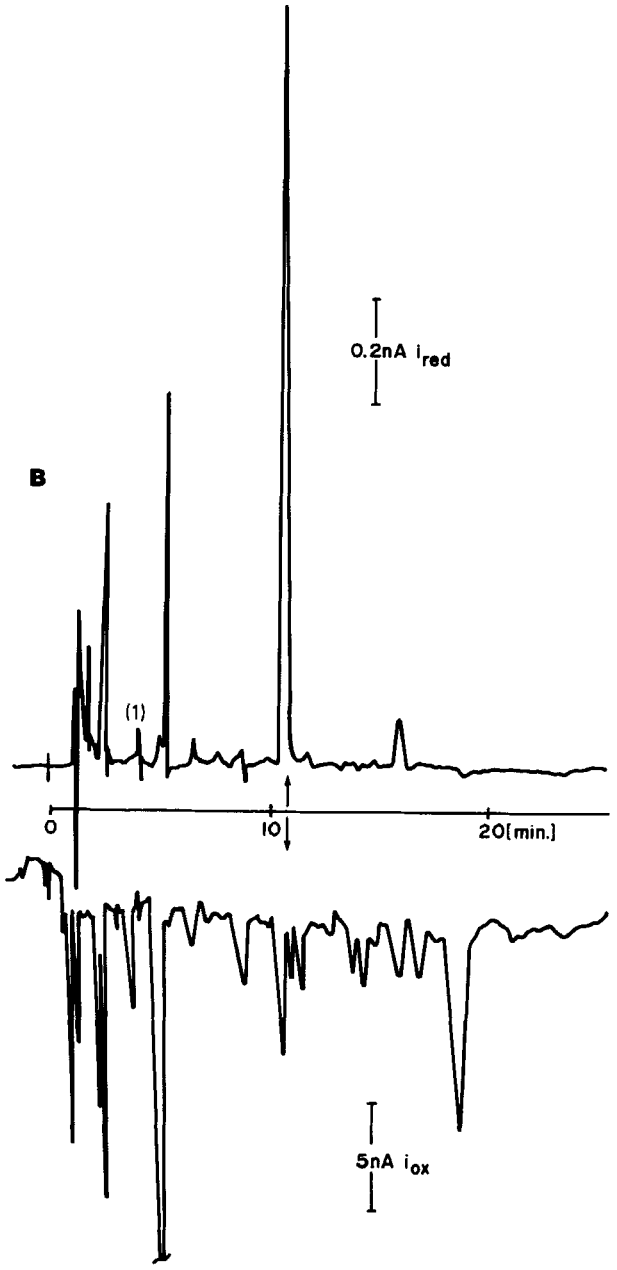

FIGURE 5B

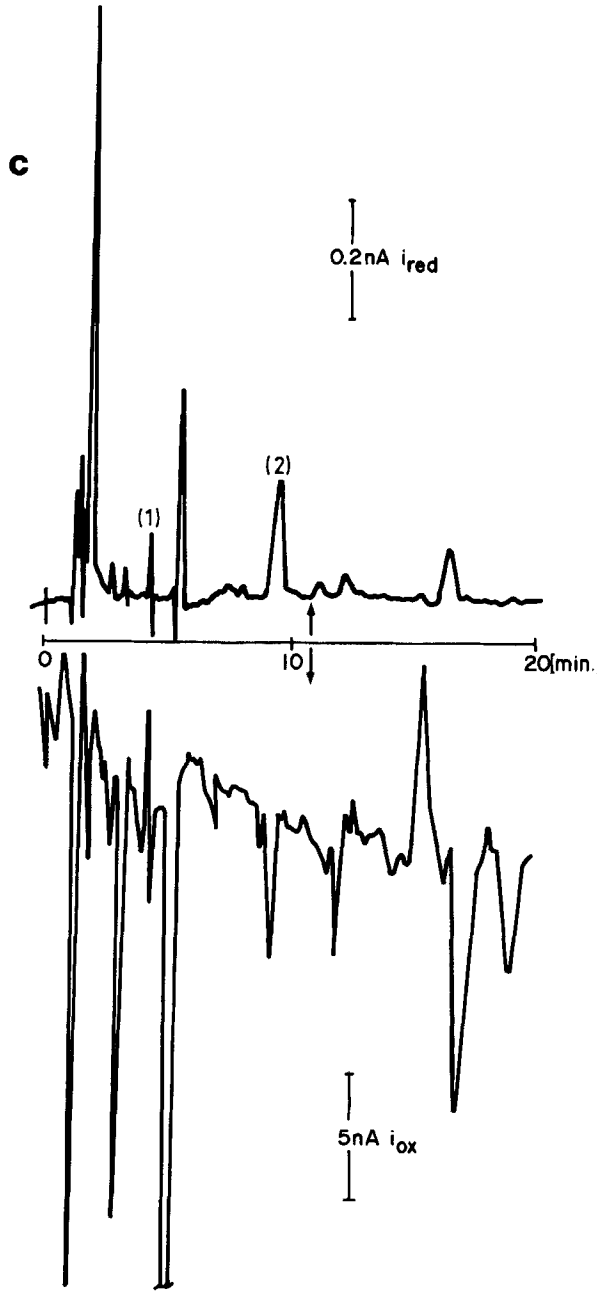

FIGURE 5C

plasma was investigated it was found to be not quite linear. At a concentration of 1 ng/mL the recovery was 35%, at 10 ng/mL it was 53% and at 100 ng/mL the recovery was 63%. This non-linearity can of course be circumvented by the use of an internal standard with the same extraction characteristics as Phy and similar electrochemical properties. Whelpton and Moore (15) e.g. used the N,N-dimethylcarbamate analogue of Phy for this purpose. Another possibility could be to use an acidic liquid-liquid extraction such the method developed by Somani and Khalique (13).

Other workers have reported on the limited stability of Phy in plasma due to enzymatic hydrolysis (15). It has been reported that addition of neostigmine to the plasma inhibits this decomposition by competing for the enzymatic reaction sites. If neostigmine was added immediately to the sample and Phy was determined within 6 hours when stored at -12°C this has been found to be a sufficient precaution. However, storage for several days is not possible as noted when samples from patients were sent to us, where only 20% of Phy remained after 3 days of storage at -20°C. This is clearly a challenging assay from biochemical, chemical and chromatographic points of view.

SUMMARY

In this assay for the determination of Phy in blood plasma we have utilized the special electrochemical properties of Phy, which were initially investigated by cyclic voltammetry. The use of series dual electrode detection where the first electrode serves as a generator of the species detected at the second electrode, offers certain advantages. The detection potential of +0.1 V made accessible by this approach, offers low background current yielding a stable baseline with improved signal-to-noise ratio, as compared to direct oxidation of Phy at +1.0 V. Furthermore, the selectivity is much improved which is highly desirable when determining trace amounts of analyte in a complex sample.

Reverse phase ion-pair chromatography was found to give symmetrical peak shape and satisfactory chromatographic separation of Phy from other plasma components as well as from its decomposition products Ese and Rub. Using the method described, where a 2 mL plasma sample is concentrated to 100 µL and 20 µL is injected, gives a detection limit for Phy of 0.5 ng/mL. Unfortunately, the solid phase extraction did not yield constant recovery in the interesting low concentration range, most likely due to adsorption. Furthermore, the limited stability of Phy in plasma due to enzymatic decomposition has to be considered for an accurate determination.

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